The Reaction of Ferrous Horseradish Peroxidase with Hydrogen Peroxide*

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SUMMARY

Hydrogen peroxide reacts with ferrous horseradish peroxidase and converts it to oxyperoxidase in a sequence of two reactions. The first is the reaction of ferrous peroxidase with H₂O₂ to form Compound II; the second is the reaction of Compound II with H₂O₂ to form oxyperoxidase.

Both reactions follow second order kinetics, being first order with respect to each of the reactants. They proceed without detectable intermediates, and, therefore, appear to be single step 2-electron oxidations.

Horseradish peroxidase is a protein with a molecular weight of about 40,000 which contains a single protoporphyrin IX heme group. This enzyme catalyzes the oxidation of a variety of substrates by hydrogen peroxide. The sequence of events involved in this catalysis is quite well understood as the result of the work of Keilin and Mann, Keilin and Hartree, Theorell, Chance, and George (see reviews by Paul (1) and Nicholls (2)).

The ferric derivative reacts with 1 mole of H₂O₂ to form Compound I, which has a formal oxidation state of +5. A 1-electron reduction converts this to Compound II with a formal oxidation state of +4, and this is converted by a second 1-electron reduction to HRP⁺³.¹

The enzyme also catalyzes the oxidation of certain substrates, notably dihydroxyfumarate (3), by molecular oxygen, and this process is only poorly understood. It had long been known that if ferrous peroxidase was exposed to oxygen, it was rapidly converted to HRP⁺³, and it was generally believed that HRP⁺³ was the only product of this reaction (3, 4). Recently, however, Wittenberg et al. (5) demonstrated that the initial product of the reaction of HRP⁺² with oxygen was a compound with absorption maxima at 417, 543, and 577 nm, and they termed the compound oxyperoxidase. The spectrum of this compound was nearly identical with that of Compound III of peroxidase, the product of the reaction of Compound II with an excess of H₂O₂. They presented considerable evidence to support the hypothesis that this was indeed an oxygenated form of peroxidase with a formal oxidation state, like that of oxyhemoglobin, of +6.

Since it is apparent that HRP⁺² can react with oxygen without being converted immediately to HRP⁺³, it was of interest to examine the reaction of ferrons HRP with other oxidizing agents. Hydrogen peroxide contains two oxidizing equivalents, and in at least one case, the conversion of HRP⁺³ to Compound I, it clearly acts as a 2-electron oxidant. Therefore, one can naively postulate that hydrogen peroxide should be able to convert HRP⁺² to oxyperoxidase in two steps, each step being a 2-electron oxidation. The intermediate in this reaction sequence should be the +4 oxidation state of HRP, Compound II.

We report here results to show that the above reaction sequence does indeed occur when HRP⁺² is mixed with H₂O₂.

Because of the complexity of the system and the multiplicity of reactions which hydrogen peroxide can potentially produce with HRP, simply mixing reactants and observing the final products is of limited utility in establishing reaction pathways. For this reason, kinetic methods have been used extensively in this study. In particular, kinetic difference spectra have been obtained and compared with spectral differences between known compounds of HRP in order to establish the initial and final states of HRP in any given kinetic process.

MATERIALS

Horseradish Peroxidase—This material was from Boehringer Mannheim and was used without further purification. Stock solutions were exhaustively dialyzed to remove all traces of ammonium sulfate.

Ferrous HRP—This was prepared by titration with dithionite under carefully controlled anaerobic conditions, as has been described previously (5).

Hydrogen Peroxide Solutions—These were prepared by diluting a stock 30% hydrogen peroxide solution into anaerobic buffer. No attempt was made to deoxygenate the stock H₂O₂ solutions so that the ratio of H₂O₂ to O₂ in such a solution should be greater than 10¹⁰. The H₂O₂ concentration was determined spectrophotometrically at 240 nm using a molar extinction coefficient of 43.6.
Reaction Kinetics—A Gibson-Milnes stopped flow apparatus (6) with a 2-cm light path in the observation cell was used for all kinetic measurements. The apparatus was equipped with an on-line computer for rapid data acquisition and processing (7).

Kinetic Difference Spectra—These were obtained by measuring the change in the millimolar extinction coefficient observed during the course of the kinetic process as a function of the wavelength of the observation light.

Static Spectra—These were determined with a Beckman model DK-2 or a Cary model 14 recording spectrophotometer.

Spectra of Various HRP Compounds—These were kindly furnished by Dr. Jonathan Wittenberg and difference spectra were computed from these.

Rapid Mixing—Solutions for static spectrophotometry were always prepared using rapid mixing methods because of the numerous side reactions possible between HRP$^{+2}$ and the higher oxidation states of the enzyme. Therefore, even when only final reaction products were to be examined, solutions were mixed by being forced through a narrow bore (1 mm) T-tube at a flow rate of approximately 10 ml per sec.

Experimental Conditions—All experiments were carried out at 20° in 0.05 M potassium phosphate buffer at pH 7.

RESULTS

Product of Reaction of HRP$^{+2}$ with Excess $\text{H}_2\text{O}_2$ as Observed by Static Spectrophotometry—HRP$^{+2}$ (0.05 mM) was rapidly mixed at 20° with an equal volume of H$_2$O$_2$ (1.25 mM) so that the H$_2$O$_2$ was present in great excess. The spectrum of the resultant solution was determined approximately 10 sec after mixing. This spectrum had maxima at 417, 545, and 577 nm and closely resembled the spectrum of oxyperoxidase or Compound III of peroxidase. With time the spectrum changed in a complex fashion, probably owing to the decay of oxyperoxidase to ferric peroxidase (5) which then reacts again with $\text{H}_2\text{O}_2$.

Kinetics of Reaction of HRP$^{+2}$ with $\text{H}_2\text{O}_2$—It seemed doubtful that oxyperoxidase could be the initial product of the reaction of HRP$^{+2}$ with $\text{H}_2\text{O}_2$. Therefore, the reaction was examined by stopped flow kinetic methods in order to determine the sequence of events involved in oxyperoxidase formation.

It is very difficult to reduce HRP$^{+3}$ stoichiometrically to HRP$^{+2}$ allowing neither residual HRP$^{+3}$ to remain in the system nor excess dithionite to be present. Since HRP$^{+4}$ reacts with H$_2$O$_2$, its presence will complicate the kinetic behavior of the system, but excess dithionite might not be innocuous, as the products of its reaction with $\text{H}_2\text{O}_2$ might also react with some forms of HRP. Therefore, the reaction was examined both with HRP reduced with an excess of dithionite and with incompletely reduced HRP containing approximately 50% HRP$^{+4}$.

Excess Dithionite—HRP$^{+4}$ reduced with a slight excess of dithionite was mixed with $\text{H}_2\text{O}_2$ so that the reactant concentrations after mixing were 0.09 mM HRP$^{+3}$ and 0.43 mM H$_2$O$_2$. A relatively rapid reaction was seen, followed by a very slow change in extinction which appeared to be more complex than a single reaction. The kinetic difference spectrum for the rapid reaction was constructed and appears in Fig. 1. The points are the changes in the millimolar extinction coefficient determined kinetically. The solid line is the difference spectrum obtained by subtracting the spectrum of HRP$^{+4}$ from the spectrum of Compound II of HRP. The agreement is quantitatively good. Since H$_2$O$_2$ was present in a 20-fold excess, the reaction was
The second order rate constant for the reaction HRP\textsuperscript{3+} with H\textsubscript{2}O\textsubscript{2} to form Compound II is plotted against the wave length of the observation light.

The points represent the kinetic difference spectrum for the slower of the reactions observed when HRP\textsuperscript{3+} is mixed with H\textsubscript{2}O\textsubscript{2}. The change in millimolar extinction coefficient occurring during the reaction is plotted as a function of the wave length of the observation light. The solid line is the difference spectrum obtained when the spectrum of Compound II of peroxidase is subtracted from that of oxyperoxidase.

treated as pseudo first order and ln (ΔOD.ₐ/ΔOD.ₕ) was plotted against time. In Fig. 2, we see that this plot is linear, indicating a first order dependence of the reaction rate on HRP\textsuperscript{3+} concentration. The rate of the reaction was measured as a function of H\textsubscript{2}O\textsubscript{2} concentration. Fig. 3 shows the linear relationship that was found, indicating a first order dependence on H\textsubscript{2}O\textsubscript{2} concentration.

As a final proof that this was a single well defined reaction, the second order rate constant was measured as a function of the wave length of the observation light beam. As it is shown in Fig. 4, the rate constant did not vary significantly over the wave length range examined.

Since HRP Compound II was not the stable product of the reaction, as observed by static spectrophotometry, it was important to demonstrate kinetically the formation of this product, oxyperoxidase. As has been mentioned, a second reaction was observed, but it was very slow and did not appear to be homogeneous at this low H\textsubscript{2}O\textsubscript{2} reactant concentration. Therefore, the H\textsubscript{2}O\textsubscript{2} concentration was raised so that after mixing with HRP\textsuperscript{3+} it was 0.046 M. At this H\textsubscript{2}O\textsubscript{2} concentration the conversion of HRP\textsuperscript{3+} to Compound II was too fast to be observed. A single reaction was observed and was of sufficiently different rates that they could be sepa-
The second reaction observed, the conversion of Compound I to Compound II, is certainly caused by the initial presence of HRP$^{++}$ in the reaction mixture. HRP$^{++}$ reacts with H$_2$O$_2$ to yield Compound I (8). This reaction, however, is so rapid that it would not be observable at the H$_2$O$_2$ concentration used in this experiment (9). The decay of Compound I to Compound II varies in rate from one HRP preparation to another. This is a 1 electron reduction, but the electron donor for this reaction is unknown.

**Discussion**

The identification by means of kinetic difference spectra of the nature of the alteration in the state of HRP taking place in any particular reaction is valid only in so far as the spectra of all of the possible states of HRP are known. There are good reasons to believe that this is the case, but it is still in the nature of an assumption and would be recognized as such. HRP can exist in five different oxidation states: +2 ferrous HRP, +3 ferric HRP, +4 Compound II (10), +5 Compound I (11), and +6 oxyperoxidase or Compound III. For two of the compounds, ferrous and ferric HRP, the oxidizing equivalents reside totally with the heme group. Therefore, these compounds can react reversibly with various ligands. In the case of the other compounds, the oxidizing equivalents probably reside partially with the ligand, and substitution of the ligand, therefore, alters the oxidation state. The assignment of a +6 oxidation level to oxyperoxidase is based upon a considerable amount of suggestive evidence, especially the spectral similarity of the compound to the oxygenated derivatives of other heme proteins, but the correctness of this assignment has not been proven. In all probability, oxyperoxidase and Compound III are one and the same. The hesitation to equate them is due to the almost complete lack of information about the properties of Compound III other than its spectrum. No other HRP compounds have been recognized, and, since there is a known compound corresponding to each oxidation state, the list would seem to be complete. Furthermore, all of the kinetic difference spectra obtained in the present study can be synthesized from the spectra of these known compounds.

From the data presented, it appears that Compound II can be formed by a pathway other than a 1-electron reduction of Compound I. Since the reaction of HRP$^{++}$ with H$_2$O$_2$ to form Compound II is first order with respect to both reactants, the simplest interpretation is that a single molecule of HRP$^{++}$ and a single molecule of H$_2$O$_2$ are involved. If this is so, and since the formal oxidation state of Compound II is known to be +4 (10), all of the oxidizing equivalents must remain with the HRP molecule, even if there is more than one product of the reaction. The structure of Compound II which is proposed by Feisach et al. (12) (HRP(heme de)-OOH)$^{IV}$ suggests that this might be simply a liganding reaction involving no change at all in the state of the iron.

The reaction of Compound II with H$_2$O$_2$ is also first order with respect to both reactants. Here the product of the reaction, oxyperoxidase, probably has a formal oxidation state of +6. Again, if a single molecule of each reactant is involved in the reaction, regardless of the number of products, all of the oxidizing equivalents must remain with the HRP molecule. If oxyperoxidase is truly an oxygenated form of peroxidase, then 2 water molecules should be produced in the course of the conversion of HRP$^{++}$ to oxyperoxidase. However, without definite knowledge...
of the structures of the HRP compounds, particularly Compound II, one cannot say during which reaction the water is formed.

No reaction intermediates were detected in either reaction, although this does not prove their absence. However, it is unlikely that compounds of intermediate oxidation state could form during these reactions unless they are forms other than those normally associated with the particular oxidation states involved. If HRP$^{+3}$ were formed initially when HRP$^{+2}$ reacts with $\text{H}_2\text{O}_2$, then it should in turn react with $\text{H}_2\text{O}_2$ to form Compound I. However, Compound I is seen only when HRP$^{+3}$ is present in the initial reaction mixture. Likewise, Compound I is an unlikely intermediate in the reaction of Compound II with $\text{H}_2\text{O}_2$ since Compound I cannot be converted to oxyperoxidase or Compound III except by being first reduced to Compound II.

Kinetic measurements cannot establish a reaction stoichiometry nor can they prove the absence of reaction intermediates. Nevertheless, the data clearly show that $\text{H}_2\text{O}_2$ converts HRP$^{+2}$ to oxyperoxidase in two sequential reactions with the intermediate formation of Compound II. Furthermore, the simplest mechanism consistent with the data is that both of these reactions are single step 2-electron oxidations, each involving a single molecule of hydrogen peroxide.

REFERENCES

8. Theorell, H., Enzymologia, 10, 250 (1941).